

STIC-ILL

Op 181. A1 E5

Adams

From: Holleran, Anne
Sent: Tuesday, September 04, 2001 6:03 PM
To: STIC-ILL
Subject: refs. for 09/251,133

Examiner: Anne Holleran
Art Unit: 1642; Rm 8E03
Phone: 308-8892
Date needed by: ASAP

Please send me copies of the following :

1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79
2. Chien, J. et al. Int. J. of Cancer (2001) 91(1): 46-54
3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
5. Rayford, W. et al. Prostate (1997) 30(3): 160-166
6. Xue-Zhang, Q. et al. Endocrine (1995) 3(6): 445-451
7. Shah, G.V. et al. Endocrinology (1994) 134(2): 596-602
8. Rayford, W. et al. J. of Urology (1994) 151(5 suppl): 490A
9. Rayford, W. et al. J. of Urology (1993) 149(4 suppl): 479A
10. Shah, G.V. et al. Prostate (N.Y.) (1992) 21(2): 87-97
11. Sagol, O. et al. Annals of Medical Sciences (1999) 8(1): 14-21
12. Sussenot, O. et al. Prostate (1998) 36(suppl. 8): 43-51
13. Hanna, F.W. et al. J. Endocrinol. (1997) 152(2): 275-281
14. Sim, S.J. et al. Annals of Clinical and Laboratory Science (1996) 26(6): 487-495
15. Watanabe, K. et al. Fukushima J. Medical Science (1995) 41(2): 141-152
16. Esik, O. et al. European J. Gynaecological Oncology (1994) 15(3): 211-216

Calcitonin Stimulates Growth of Human Prostate Cancer Cells through Receptor-Mediated Increase in Cyclic Adenosine 3',5'-Monophosphates and Cytoplasmic Ca²⁺ Transients*

GIRISH V. SHAH, WALTER RAYFORD, MARK J. NOBLE, MARK AUSTENFELD, JOHN WEIGEL, SUSAN VAMOS, AND WINSTON K. MEBUST

Departments of Urologic Surgery (G.V.S., M.J.N., W.R., M.A., J.W., W.K.M.) and Physiology (G.V.S.), University of Kansas Medical Center, Kansas City, Kansas 66103; and the Kansas City Veterans Administration Medical Center (G.V.S., M.J.N., S.V.), Kansas City, Missouri 64128

ABSTRACT

Our recent study has shown that a calcitonin (CT)-like immunoreactive substance(s) is secreted by cultured prostate cells, and secretion of this material is significantly higher in malignant than in benign prostate cells. To test the hypothesis that prostatic CT may serve as a paracrine/neuroendocrine factor, the present study investigated for the presence of CT receptors in the prostate gland. Signal transduction mechanisms activated by CT were examined, and the study also tested its effects on prostate cell proliferation, as assessed by [³H]thymidine incorporation. The results show that high affinity binding sites for [¹²⁵I]salmon CT were present in plasma membrane fractions of human prostate tissue specimens and the prostate cancer LnCaP cell line. The maximal binding for CT receptors was 564 ± 163 fmol/mg protein, and the apparent dissociation constant (K_d) was 2.89 ± 0.58 nM. CT induced a dose-dependent increase in cAMP generation in LnCaP cells. The

effect of CT on cytoplasmic Ca²⁺ transients of LnCaP cells was examined by videofluoromicroscopy. CT (100 nM) induced a rapid and sharp increase in cytoplasmic Ca²⁺ concentrations in LnCaP cells. The CT-induced increase in cytoplasmic Ca²⁺ transients appeared to be biphasic (spike and plateau), and this increase was 4- to 10-fold during the initial phase. The profile of this response is characteristic of the activated Ca²⁺/phospholipid second messenger system. CT also caused a dose-dependent increase in [³H]thymidine incorporation by LnCaP cells. These results suggest that a locally secreted CT-like peptide(s) induces mitogenic responses in prostate cancer cells. This action seems to be mediated through activation of signaling mechanisms, leading to the accumulation of two different second messengers, cAMP and calcium. Activation of dual second messenger systems by CT receptors suggests that the peptide hormone may play an important role in rapidly growing cell populations during the process of tumor formation. (Endocrinology 134: 596-602, 1994)

THE HUMAN prostate gland contains a significant population of neuroendocrine cells, and these cells secrete neuropeptides, such as bombesin, serotonin, and TSH-like, PTH-like, and calcitonin (CT)-like peptides (1-3). As many of these peptides also act as mitogens in peripheral organs, it is conceivable that they may participate in the differentiation and growth of the prostate gland in concert with androgens (4-6). Accumulating evidence suggests that the relative population of neuroendocrine cells, specifically that of CT-immunopositive cells, seems to markedly increase in prostatic adenocarcinoma (6, 7). Recent findings from this laboratory have shown that an immunoreactive salmon (s) CT-like product(s) is secreted by cultured human prostate cells, and relative secretion of this material is significantly higher in cancer than benign cells (2). These results raise the possibility that an elevated expression of CT-like products, such as a sCT-like peptide(s) in the prostate gland may be associated with increased growth and/or tumor development.

Elevated secretion of a CT-like ectopic peptide(s) has been

reported in several human tumors, such as breast, renal, lung, and gastric carcinoma, and their specific binding sites have also been identified in these organs (7-15). The present studies investigated for the presence of high affinity CT-binding sites in tissue specimens obtained from patients undergoing prostate surgeries. Membranes prepared from prostate cancer LnCaP cells were also tested for the presence of CT receptors, because these cells have been used as an *in vitro* model to study the control of prostate cell proliferation by androgens and growth factors (16). This cell line has been derived from a lymph node of a subject with metastatic carcinoma of the prostate, and like the prostate gland, these cells are extremely sensitive to androgens. Deprivation of androgens drastically reduces the proliferative as well as secretory activity of LnCaP cells, and proliferative activity and DNA synthesis can be induced by the addition of testosterone and/or epidermal growth factor in the culture medium (17). LnCaP cells secrete markers of prostatic secretions, such as prostate-specific antigen and prostate acid phosphatase; however, the secretion of a CT-like product(s) or other neuroendocrine substances by these cells has not been reported (16).

Recent evidence has shown that an ectopically produced CT(s) may serve as a paracrine growth factor/growth regulator in gastric and renal carcinomas, as the peptide hormone

Received June 25, 1993.

Address all correspondence and requests for reprints to: Dr. Girish V. Shah, Department of Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66103.

*This work was supported by NIH Grant DK-45044 and a Scientific Educational Partnership Foundation grant from the Marion Merrel Dow Foundation (to G.V.S.).

modulates the proliferation of these cells by activating different signaling mechanisms (12, 13). To test whether prostatic CT may have a similar autocrine/paracrine role in prostate function, the present study investigated the effects of CT on DNA synthesis in cultured LnCaP cells. Proliferative actions of CT were compared with those of other known mitogens and pharmacological agents, such as dihydrotestosterone (DHT), insulin, phorbol myristic acid (PMA), and (Bu)₂cAMP (dbcAMP). Additional studies investigated the effects of CT on cAMP accumulation and cytoplasmic Ca^{2+} transients in cultured LnCaP cells.

Materials and Methods

Peptides and chemicals

Synthetic CT peptides (salmon and human) were obtained from Peninsula Laboratories (Thousand Oaks, CA). Aprotinin and leupeptin were purchased from Boehringer Mannheim (Indianapolis, IN). Carrier-free sodium [¹²⁵I]iodide was obtained from Radiochemical Center, Amersham (Aylesbury, Buckinghamshire, United Kingdom). Porcine insulin was kindly provided by Nova Laboratories of Novo Nordisk Pharmaceutical, Inc. (Princeton, NJ). Reagents for tissue culture, media, and sera, such as Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640, and bovine and fetal calf sera, were obtained from Gibco-BRL (Grand Island, NY). dbcAMP, PMA, DHT, and all other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture

A human metastatic prostate adenocarcinoma cell line (LnCaP; American Type Culture Collection, CRL 1740) was obtained from American Type Culture Collection (Baltimore, MD), and the cells were propagated in a culture medium (consisting of RPMI-1640 supplemented with L-glutamine, 5% fetal calf serum, 15% newborn calf serum, 10 mM HEPES, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin) at 37°C in a modified atmosphere containing 95% air and 5% CO₂ (2). Cell cultures near confluence were used in all experiments unless otherwise noted.

[¹²⁵I]sCT receptor binding assay

Human prostatic tissue specimens were obtained from biopsies or surgeries performed at the University of Kansas Medical Center. The age of this patient population ranged from 48–71 yr (mean age, 63 yr). Appropriate informed consent was obtained in all cases.

Preparation of membranes. Human prostate tissue specimens or confluent cultures of LnCaP cells were processed to obtain the membrane fractions, described by Kadar *et al.* (18). In brief, tissue specimens or LnCaP cells were washed with PBS and homogenized individually in 10 vol of a lysis buffer (25 mM Tris-HCl, pH 7.5, containing 0.3 M sucrose, 0.5 mM EDTA, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 50 $\mu\text{g}/\text{ml}$ bacitracin). The homogenates were centrifuged at 1,000 rpm for 10 min at 4°C, and the supernatants were centrifuged again at 35,000 $\times g$ for 1 h at 4°C to obtain the membrane fractions. The membrane pellets were resuspended in the suspension buffer (25 mM Tris-HCl, pH 7.4; 5 mM MgCl₂; 5 $\mu\text{g}/\text{ml}$ aprotinin; 5 $\mu\text{g}/\text{ml}$ leupeptin; 50 $\mu\text{g}/\text{ml}$ bacitracin; 0.25 mM phenylmethylsulfonylfluoride) and recentrifuged at 35,000 $\times g$ for an additional 60 min at 4°C. The final pellet was resuspended in the suspension buffer, and its protein content was determined by the bicinchoninic acid method (19). The plasma membrane suspensions were aliquoted and stored frozen at –80°C.

Receptor binding. Synthetic sCT was iodinated to a high specific activity using 1 mCi Na¹²⁵I and 1 μg chloramine-T. The reaction was allowed to proceed for 10 sec and was stopped by the addition of 10 μl of metabisulfite (1 mg/ml). Typically, the specific activity of the

resulting [¹²⁵I]sCT varied from 200–300 Ci/mmol. The radiolabeled peptide was purified by gel permeation chromatography on a Sephadex G-25 (fine) column (1 × 50 cm) at 4°C. Ammonium acetate (0.2 M; pH 4.7; containing 0.25% BSA) was used as an eluent. Purified [¹²⁵I]sCT fraction was mixed with acetone (20%, by vol) and acetic acid (1%, by vol), and stored in small aliquots at –80°C.

The prostate plasma membranes (30–40 μg protein), [¹²⁵I]sCT, and various concentrations of cold ligands [sCT or human (h) CT] were diluted in the assay buffer (25 mM Tris-HCl, pH 7.4; 5 mM MgCl₂; 0.1% BSA; 5 $\mu\text{g}/\text{ml}$ aprotinin; 5 $\mu\text{g}/\text{ml}$ leupeptin; 50 $\mu\text{g}/\text{ml}$ bacitracin; and 0.25 mM phenylmethylsulfonylfluoride) and incubated in a final volume of 0.25 ml at 0°C (on ice) for 2 h. At the end of the incubation, the reaction mixture was carefully layered on 1 ml wash buffer (10 mM Tris-HCl, pH 7.4; 100 mM choline chloride; and 0.1% BSA) in separate 1.5-ml tubes and centrifuged at 13,000 $\times g$ for 30 min. The supernatant was carefully aspirated, the tube bottoms were cut, and the radioactivity in sedimented pellets was measured in a Beckman Autogamma Counter (Palo Alto, CA). The incubation mixture in a saturation assay contained 30–40 μg membrane protein/tube and increasing amounts of [¹²⁵I]sCT (50–1,000 fmol) in the absence and presence of 1 μM unlabeled sCT. In a competition assay, the membranes (30–40 μg protein) were incubated with [¹²⁵I]sCT (100–120 fmol) and increasing concentrations of unlabeled sCT or hCT (0–200 nM). Each treatment was repeated three or more times.

The binding data obtained from the assays were analyzed with the Ligand-PC computer program (20), and the binding constants of [¹²⁵I]sCT-binding sites were determined. The results are presented as the mean ± SEM apparent dissociation constant (K_d) and maximal binding (B_{max}).

cAMP measurements

For measurement of cAMP accumulation, LnCaP cells were cultured to subconfluence in polylysine-coated six-well culture plates. The attached cells were washed and preincubated for 2 h in a serum-free basal medium (serum- and bicarbonate-free RPMI-1640 medium containing 10 mM HEPES, pH 7.4; 0.3% BSA; 280 $\mu\text{g}/\text{ml}$ bacitracin; and 10 $\mu\text{g}/\text{ml}$ gentamicin), washed, and treated with increasing concentrations of sCT (0.1 pM to 1 μM) in the presence of 10 μM 3-isobutyl-1-methylxanthine for 10 min at 37°C. Each treatment was performed in triplicate. At the end of the incubation, medium was replaced with 1 ml sodium acetate buffer (pH 4.8), and the cells were frozen at –80°C, thawed, scraped, and transferred to a 1.5-ml tube. The cell lysates were frozen again, thawed, and centrifuged at 4°C to remove insoluble materials, and the supernatants were stored frozen until their cAMP contents were analyzed by RIA. The cAMP RIA used [¹²⁵I]2'-O-monosuccinyl-cAMP tyrosyl methyl ester as a tracer, and anti-cAMP rabbit serum was purchased from Biomedical Technologies (Boston, MA). The lower limit of detection for the assay was 0.08 pmol/ml, and the intraassay coefficient of variation among controls was less than 6%. Each sample was analyzed in duplicate, and all samples from each experiment were analyzed in a single assay to avoid interassay variations.

The average basal cAMP concentration was 350 fmol/well. Data are expressed as mean femtomoles of cAMP accumulated ± SEM. The results were statistically evaluated by one-way analysis of variance, and the level of significance was derived from Duncan's multiple range test.

Measurements of cytoplasmic Ca^{2+} transients

LnCaP cells were grown to subconfluence on coverslips (size, 1 in.), as described in *Cell culture* above. When ready, the cells were washed with serum-free DMEM and loaded with the fluorescent dye indo-1AM ester (5 μM ; Molecular Probes, Eugene, OR) in the presence of the detergent Pluronic F127 (0.34 $\mu\text{g}/\text{ml}$; Molecular Probes) in a serum-free DMEM for 1 h at 37°C. The cells were rinsed with serum-free DMEM and then maintained in DMEM containing 15% bovine calf serum and 5% fetal calf serum for 1 h at 37°C before the experiment.

Simultaneous fluorescence measurements from 3–4 individual cells were made using 4-channel videofluorescence microscopy (21). Coverslips carrying the cells were placed in 1 ml Ringer's solution (145 mM NaCl, 2.5 mM K₂HPO₄, 10 mM HEPES, 1 mM CaCl₂, 1 mM MgSO₄, 10

mm glucose, and 0.3% BSA, pH 7.4) in a thermostatted microscope stage perfusion chamber which was maintained at 37°C. The cells were excited by a Nikon Xenon lamp on the stage of Nikon Diaphot microscope. The emitted fluorescent images at 405 and 475 nm were recorded on intensified CCD cameras (KS-1381 Videoscope, Washington DC) after splitting the signal with dichroic mirrors (445 and 510 nm). The images were typically acquired for 10 min and recorded onto a 400-line resolution video recorder with the time code generator/reader (VO 9600 U-Matic, SONY, Inc., Teaneck, NJ) for additional off-line analysis. Usually, data from 12–18 individual cells were collected during each experiment.

The ratios of fluorescence at 405 and 475 nm were calculated as a function of time using computer software (Micromeasure IM-4000, Belvoir Consulting, Long Beach, CA), and intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) was determined as described previously (21). The results were plotted as nanomoles of $[\text{Ca}^{2+}]_i$ as a function of time.

^3H Thymidine incorporation assay

Cultured LnCaP cells in log phase were used to seed polylysine-coated 24-well culture plates at a density of 3×10^4 cells/well (Falcon Plastics, Oxnard, CA) and cultured overnight (16–20 h) in 1 ml culture medium (as described in *Cell culture* above). The proliferation rate of the cultured cells was slowed by incubations, first in a low serum medium (RPMI-1640 containing 3% fetal calf serum) for 16 h, then in serum-free basal medium (RPMI-1640 containing 0.3% BSA, 10 mM HEPES, 280 $\mu\text{g}/\text{ml}$ bacitracin, and antibiotics) for 2 h. At this point, the medium was replaced with fresh serum-free basal medium containing various concentrations of CT (0–1000 nM), DHT (10 pM), insulin (10 nM), PMA (1 nM), and dbcAMP (0.2 μM) for 24 h. The cells also received ^3H thymidine (1 $\mu\text{Ci}/\text{well}$) 4 h before termination of the assay. At the end of the incubation, the cells were washed twice with ice-cold PBS (containing 100 μM unlabeled thymidine) and solubilized in Triton X-100 (0.1%, vol/vol; in distilled water). The samples were added to a liquid scintillant (Fisher Scientific, Pittsburgh, PA), and the incorporated ^3H thymidine was quantified in a Beckman liquid scintillation counter (Palo Alto, CA).

The results are expressed as mean counts per min of ^3H thymidine incorporated \pm SEM. Data were analyzed by one-way analysis of variance or Student's *t* test, and the levels of significance were derived from Duncan's multiple range test or a two-tailed table, respectively.

Results

Binding of $[^{125}\text{I}]s\text{CT}$ to prostate plasma membranes

The specific binding of $[^{125}\text{I}]s\text{CT}$ to prostate membranes (total binding – nonspecific binding) was saturable and linear with protein concentrations of 5–30 μg protein/tube (data not shown).

$s\text{CT}$ -binding sites of prostate membranes and LnCaP cells were characterized in saturation and competition assays. The data from saturation assays suggest that the binding of $[^{125}\text{I}]s\text{CT}$ to prostate membranes was saturable (Fig. 1A). Scatchard analysis of the binding data from saturation assays yielded linear curves, suggesting a single class of binding sites (Fig. 1B). However, the presence of additional classes of binding sites could not be excluded. The B_{\max} was 564 ± 163 fmol/mg protein, and the apparent K_d was 2.89 ± 0.58 nM ($n = 8$). Prostate membranes from seven individual tissue specimens (four benign prostatic neoplasia, three carcinoma, and LnCaP cells) were analyzed, and the results are presented in Table 1. No significant differences in binding constants between the benign prostatic neoplasia and cancer specimens were observed. The ability of unlabeled $s\text{CT}$ and $h\text{CT}$ peptides to displace $[^{125}\text{I}]s\text{CT}$ binding was tested in competition assays. As has been reported for CT receptors in

other tissues, the present results demonstrate that $h\text{CT}$ was much less effective in displacing $[^{125}\text{I}]s\text{CT}$ than $s\text{CT}$ (Fig. 2). The concentration of $s\text{CT}$ required for half-maximal inhibition of $[^{125}\text{I}]s\text{CT}$ binding was 0.94 nM. At the highest tested concentration of 200 nM, $h\text{CT}$ could reduce $[^{125}\text{I}]s\text{CT}$ binding by only 20%.

Effect of CT on cAMP accumulation

The effect of CT on cAMP generation was tested at increasing concentrations from 1 pM to 1 μM . The peptide hormone caused a dose-related increase in cAMP generation in cultured LnCaP cells up to a concentration of 1 nM. Further increases in CT concentrations did not produce any additional appreciable increase in cAMP concentrations. CT (1 nM) caused a 320% increase in cAMP concentrations. The median effective concentration (EC_{50}) of CT for stimulation of cAMP generation was 0.03 nM (Fig. 3). Isoproterenol was used as a reference agonist, and at 1 μM , it caused a 60-fold increase in cAMP concentrations (data not shown).

Effect of CT on cytoplasmic Ca^{2+} transients

A typical response of LnCaP cells to 100 nM CT and ionomycin is depicted in Fig. 4. The responding cells displayed a transient, rapid, spike and plateau increase in their $[\text{Ca}^{2+}]_i$. Typically, a 4- to 10-fold increase in $[\text{Ca}^{2+}]_i$ was observed within 10 sec of the stimulus. This was followed by a gradual decline to a nadir over a period of 130 sec. At this point, a secondary, but much smaller, increase in $[\text{Ca}^{2+}]_i$ was observed. This profile is similar to that of the previously reported phospholipase-C-induced increases in cytoplasmic Ca^{2+} levels (22). The cells that responded to CT were subjected to a second stimulation with 500 nM ionomycin, and as expected, they responded by showing an even larger (30- to 50-fold) increase in their cytoplasmic Ca^{2+} concentrations. CT, when tested at 1 nM, did not cause any appreciable increase in $[\text{Ca}^{2+}]_i$ (data not shown).

The permeabilization of cells during the dye-loading process seems to have affected the viability and/or responsiveness of a significant LnCaP cell population. Typically, only 6–40% of the total cells responded to agonists such as CT, ionomycin, or carbachol. Thus, the results depicted in Fig. 4 are representative of the responding cell population.

Effect of CT on ^3H thymidine incorporation

Results from at least six independent experiments suggest that CT induced a significant and dose-dependent increase in ^3H thymidine uptake by LnCaP cells. Figure 5 presents a typical dose response of ^3H thymidine uptake to CT. The CT-induced increase in ^3H thymidine incorporation was statistically significant at concentrations of 10 and 100 nM, and a maximal increase of 65% was observed at 100 nM. The effect of CT on DNA synthesis may have peaked at this concentration, as a higher CT concentration (1 μM) attenuated this increase. Figure 6 depicts the effects of various mitogens on DNA synthesis in LnCaP cells. The cells were incubated with the agents for 24 h. Both DHT (10 pM) and CT (100 nM) produced a significant increase of approximately 60–70% in

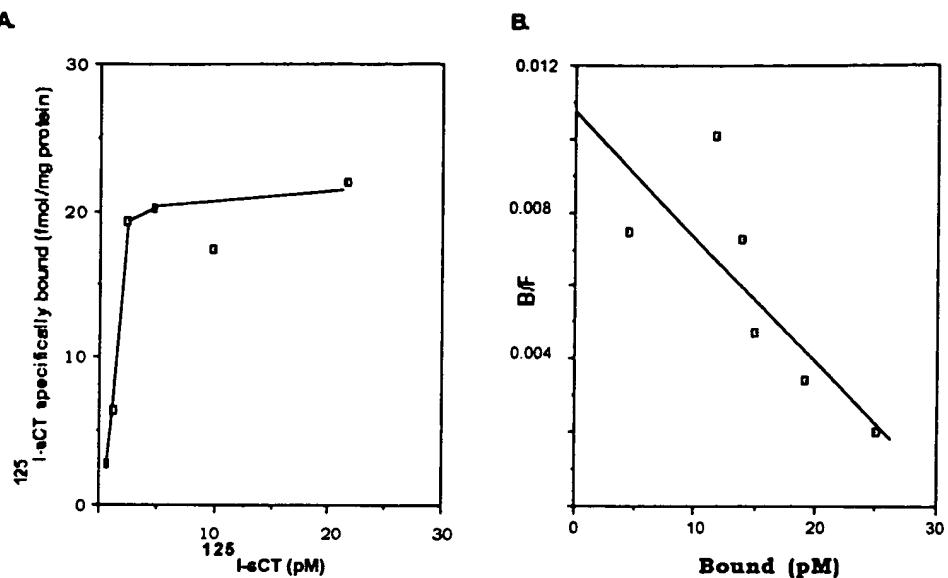


FIG. 1. Saturation analysis of [¹²⁵I]sCT binding to prostate membranes. A, Prostate membranes (30–40 µg protein/tube) were incubated with increasing concentrations of [¹²⁵I]sCT in the absence (total) or presence (nsb) of 1 µM unlabeled sCT for 2 h at 0°C. Bound and free radioactivities were separated as described in Materials and Methods. Data points represent specific binding (total – nsb). B, Scatchard analysis of specific binding data from A.

TABLE 1. CT receptor characteristics

No.	Membranes from	K _d (nM)	B _{max} (fmol/mg protein)	No. of determinations
1.	BPH	1.21 ± 0.40 ^a	408 ± 93 ^a	4
2.	CaP	3.13 ± 1.16	730 ± 194	3
3.	LnCaP	3.89	229	1

Mean ± SEM.

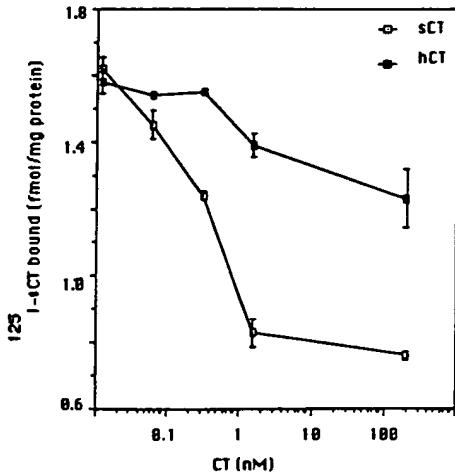


FIG. 2. Specificity of CT binding to prostate membranes. Prostate membranes were incubated with approximately 100 fM [¹²⁵I]sCT with varying concentrations of unlabeled sCT and hCT. Bound radioactivity was estimated as described in Materials and Methods.

[³H]thymidine incorporation. dbcAMP (0.2 µM) caused an approximately 50% increase in [³H]thymidine uptake, and 1 µM PMA induced an almost 2-fold increase over the unstimulated control value. Insulin (100 nM) did not affect DNA synthesis in LnCaP cells.

Discussion

Recent evidence has shown that CT-like neuroendocrine peptides, such as immunoreactive sCT-like peptide and CT

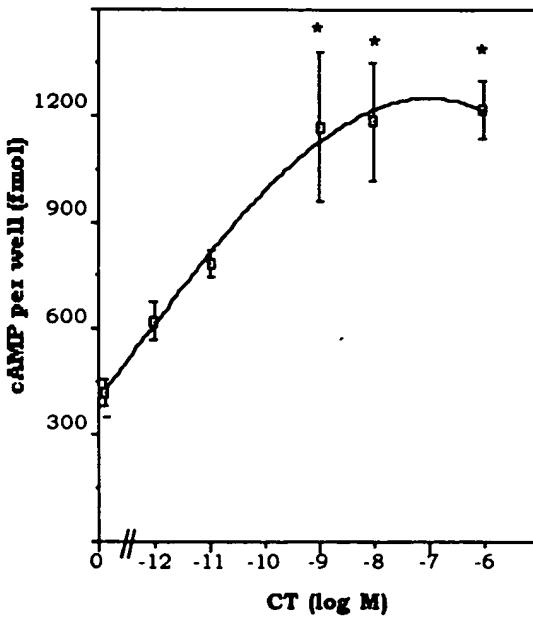


FIG. 3. Effect of CT on cAMP accumulation in LnCaP cells. Increasing concentrations of sCT were used to stimulate cultured LnCaP cells as described in Materials and Methods. Each point was measured in triplicate, and the results are presented as the mean cAMP (femtomoles per well) ± SEM (error bars) for each treatment. The cells were incubated with sCT for 10 min at 37°C in the presence of 10 µM 3-isobutyl-1-methylxanthine, then lysed, and the supernatants were assayed in duplicate with cAMP RIA. The data are expressed as mean femtomoles of cAMP produced per well ± SEM. CT induced a dose-dependent increase in cAMP generation, and the increase was statistically significant at concentrations of 1 nM and higher. *, P < 0.01 (by one-way analysis of variance and Duncan's multiple range test).

gene-related peptide, may originate within the prostate gland (1, 2, 23). However, the role of these peptide(s) in prostate physiology has not been identified. The present results demonstrate the presence of CT-binding sites in the plasma membrane fraction of human prostate tissue specimens and prostate cancer LnCaP cells. The binding of [¹²⁵I]sCT to

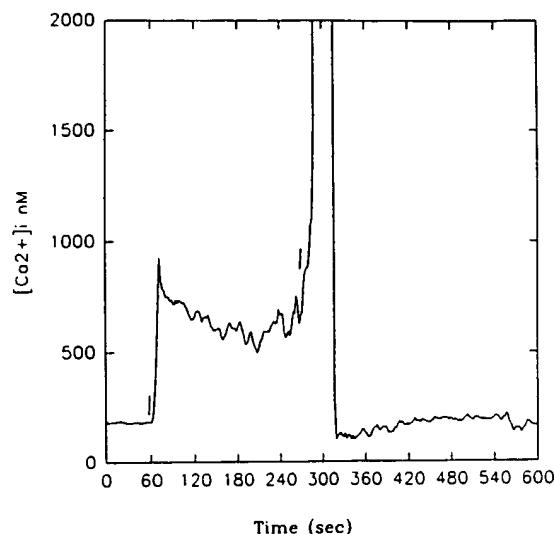


FIG. 4. Effect of CT on $[Ca^{2+}]_i$ in LnCaP cells. LnCaP cells were cultured on coverslips, and their $[Ca^{2+}]_i$ values were measured by four-channel videofluoromicroscopy as described in *Materials and Methods*. Representative trace for a responding cell is shown. The first vertical line indicates the time of 100 nM sCT addition (60 sec); a second vertical line indicates the time of 500 nM ionomycin addition (270 sec). The scale of the spectrum was changed at 285 sec, because the addition of ionomycin caused a very large increase in $[Ca^{2+}]_i$ (out of range). This experiment was repeated three times with different cultures of LnCaP and primary prostate cells, and similar responses were obtained. At each time, 4–40% of the cells responded to the agonists.

prostatic plasma membranes produced linear Scatchard plots. The mean apparent dissociation constant (K_d) of these receptors was less than 3 nM, similar to the reported K_d values for high affinity CT receptors in other tissues, such as osteoclasts; lymphocytes; kidney, brain, and liver cells; and several human carcinoma cell lines (9, 11, 23–29). The present results did not identify any significant differences in binding characteristics of CT receptors of benign and malignant prostates. As the number of samples examined in the present study was small, an expanded study will be necessary to further evaluate this question. Like CT receptors in these organs, prostate CT receptors also exhibited a higher affinity for salmon CT molecule than the hCT peptide. These results may suggest that a population of prostate CT-binding sites may not identify hCT molecules and may be specific for sCT-like endogenous ligand. The presence of such sCT receptors that are distinct from hCT and CT gene-related peptide-binding sites has been reported in rat brain (30), and genes for these receptors have recently been cloned in rat brain and skeletal muscles (31). It is conceivable that human neuroendocrine tissues may have similar distinct sCT receptors. These results, when considered with our previous finding that an immunoreactive sCT-like product(s) is secreted by cultured primary prostate cells, raise the possibility that prostate-derived CT(s) may serve as a paracrine/autocrine factor in the prostate gland.

The presence of CT receptors in LnCaP cells suggest that these cells may serve as model target cells to examine possible roles for CT(s) in prostatic carcinoma. Most cellular actions of CT are associated with activation of adenylate cyclase, as

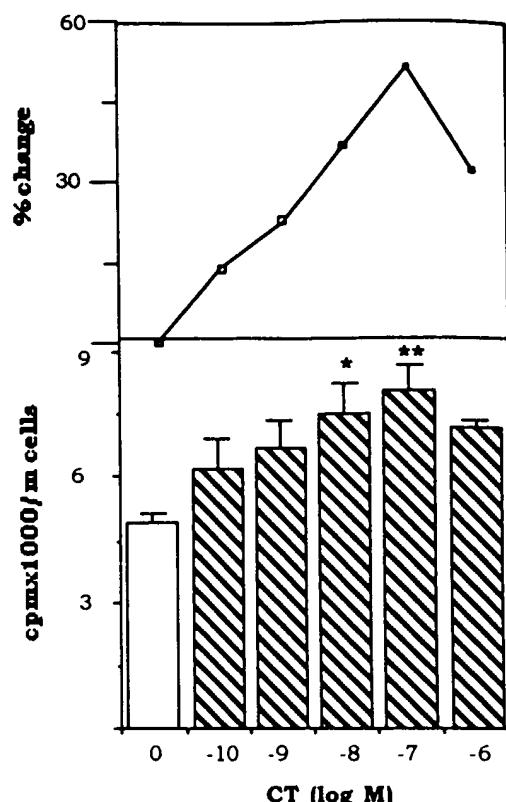


FIG. 5. Effect of CT on DNA synthesis: dose-response curve. Proliferation of LnCaP cells was examined as assessed by [3 H]thymidine incorporation after stimulation with sCT (-10 to -6 M). The cells were cultured in low serum (3% fetal calf serum-RPMI-1640) for 24 h and serum starved for 2 h before the stimulation. The cells were stimulated with the peptide hormone for 24 h and received 1 μ Ci [3 H]thymidine during the last 4 h of stimulation. The cells were lysed, and the radioactivity was determined. The data are representative of at least six separate experiments and are expressed as counts per min/1000 million cells (mean \pm SEM of sextuplicates; *lower panel*) and as the percent increase (means from the *lower panel*) with respect to the control value. sCT significantly increased DNA synthesis in LnCaP cells at concentrations of 10 and 100 nM. hCT also gave a similar, but smaller, response. *, $P < 0.01$ (by one-way analysis of variance and Duncan's multiple range test).

reported in osteoclasts, renal cells, and a majority of human carcinoma cell lines (11–13, 26–29). However, CT has also been shown to act independent of cAMP-mediated mechanisms. For example, CT elevates cytosolic Ca^{2+} in rabbit renal tubules and rat osteoclasts (32, 33). In isolated rat osteoclasts and the kidney cell line LLC-PK1, CT activates either adenylate cyclase or protein kinase-C in a cell cycle-dependent manner (13, 32). A recent study has demonstrated that the recombinant CT receptor independently stimulates cAMP- and Ca^{2+} /inositol phosphate-signaling pathways (22). The present results have shown that CT increases both cAMP generation and cytosolic Ca^{2+} concentrations in cultured LnCaP cells. The stimulatory effect of CT on cAMP generation was dose related. The median effective concentration of CT (EC_{50}) for this action was 0.03 nM, which is much lower than the apparent K_d of 2.89 nM. Thus, the EC_{50} may reflect a small fraction of CT receptors that may have been coupled to a GTP binding stimulatory (G_s) proteins. A subpopulation

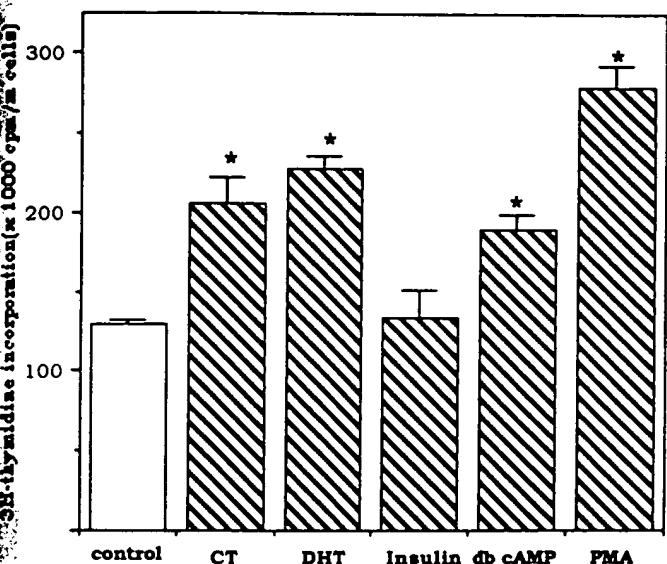


FIG. 6. The effects of various mitogens on DNA synthesis in LnCaP cells. Effects of sCT (100 nM), DHT (10 pM), insulin (1 μ M), dbcAMP (0.2 μ M), and PMA (1 nM) on [³H]thymidine incorporation were examined. The data are expressed as mean counts per min ($\times 1000$)/million cells \pm SEM of quadruples. CT, DHT, dbcAMP, and PMA caused a significant increase in [³H]thymidine incorporation in LnCaP cells. *, $P < 0.01$ (by *t* test).

of LnCaP cells responded to CT with a biphasic, spike and plateau, increase in cytosolic Ca^{2+} . However, this stimulus was observed at a higher CT concentration (100 nM). These results raise the possibility of two separate populations of CT receptors that may be coupled to Ca^{2+} /phospholipid and adenylate cyclase second messenger systems, and each of the receptor populations may be activated by a certain concentration range of CT. Alternatively, certain concentration ranges of CT may modulate coupling of its receptors to the signaling mechanisms. This is not surprising, as CT has been shown to generate different intracellular responses even in the same cells when used either at different concentrations (34) or under different physiological conditions (13, 35).

The functional significance of CT receptors in prostate membranes is not known, but their presence in the prostate gland suggests that this organ may respond to CTs that have either been produced within the prostate gland or acquired from the circulation. It is also likely that components of prostatic and systemic CTs that activate these receptors may vary with clinical or physiological conditions. Whatever the origin of CT, the present results have shown that the peptide hormone induces a dose-dependent increase in [³H]thymidine incorporation in LnCaP cells. However, a significant increase in DNA synthesis was observed only at concentrations of CT higher than the apparent K_d (10 and 100 nM). Both dbcAMP and phorbol ester also induced a significant 2-fold increase in DNA synthesis. As CT could induce DNA synthesis only at concentrations of 10 and 100 nM, it is likely that activation of both phospholipase-C- and adenylate cyclase-mediated signaling mechanisms may be necessary for CT-induced DNA synthesis. A complete chain of events associated with this process has not been elucidated, but neuroendocrine peptides have been shown to stimulate

growth and DNA synthesis through prolonged stimulation of receptors and activation of multiple signaling pathways in Swiss 3T3 cells and other target tissues (36, 37). It is likely that CT may follow a similar pathway in LnCaP cells. Additional approaches will be necessary to further examine this phenomenon.

It is becoming increasingly recognized that small regulatory peptides of neuroendocrine origin may serve as mitogens in various peripheral tissues. Specifically, bombesin, bradykinin, calcitonin gene-related peptide, and calcitonin have been shown to act as growth factors and stimulate cell proliferation in several cell types (3, 4). Ectopic and elevated expression of these peptides in several malignant organs raises the possibility that they may serve as paracrine/autocrine growth regulators during rapid growth and tumorigenesis in these organs (3–7). Recent findings from this laboratory have shown that a immunoreactive CT-like substance(s) is secreted by prostate cells in significant amounts (1, 2). When these results are considered with the present findings that high affinity binding sites for CT are present in the prostate gland and that CT induces an increase in cAMP accumulation, cytoplasmic Ca^{2+} concentrations, and [³H]thymidine incorporation in prostate cancer cells, it seems likely that a prostate-derived CT-like peptide(s) may be involved in a variety of physiological and pathophysiological events, such as growth, differentiation, and tumorigenesis. Further understanding of the secretion and role of CT in the prostate microenvironment will lead to an increase in our understanding of the factors that regulate the progression of prostate neoplasms.

Acknowledgments

We thank Mr. Benjamin Frey for expert technical assistance, and Dr. S. Bahouth for providing [¹²⁵I]cAMP for the cAMP RIA. We gratefully acknowledge Drs. Larry Welling and Thomas Wiegmann for allowing the use of their equipment for the measurement of cytoplasmic Ca^{2+} transients.

References

- di Sant'Agnese PA 1992 Neuroendocrine differentiation in carcinoma of the prostate: diagnostic, prognostic and therapeutic implications. *Cancer* [Suppl 1] 70:254–268
- Shah GV, Noble MJ, Austenfeld M, Weigel J, Deftous LJ, Mebust WK 1992 Presence of calcitonin-like immunoreactivity (iCT) in human prostate gland: evidence for iCT secretion by cultured prostate cells. *Prostate* 21:87–97
- Iwamura M, di Sant'Agnese PA, Wu G, Benning CM, Cockett AT, Deftous LJ, Abrahamsson PA 1993 Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res* 53:1724–1726
- Dalsgaard CJ, Hultgardh-Nilsson A, Haegerstrand A, Nilsson J 1989 Neuropeptides as growth factors. Possible roles in human diseases. *Regul Peptides* 25:1–9
- Rozengurt E 1991 Neuropeptides as cellular growth factors: role of multiple signaling pathways. *Eur J Clin Invest* 21:123–134
- Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, Grimelius L 1987 Peptide hormone- and serotonin-immunoreactive tumor cells in carcinoma of the prostate. *Pathol Res Pract* 182:298–307
- Imura H 1980 Ectopic hormone production viewed as an abnormality in regulation of gene expression. *Adv Cancer Res* 33:39–75
- Austin LA, Heath H 1981 Calcitonin physiology and pathophysiology.

- ology. *N Engl J Med* 304:269-278
9. Martin TJ, Findlay DM, Michaelangeli VP, Moseley JM, Eisman JA, MacIntyre I. Significance of calcitonin production, action in cancer. In: Cumming IA, Funder JW, Mendelsohn FAO (eds) *Endocrinology*. Elsevier, Amsterdam, 1980, p 657
 10. Findlay DM, Michaelangeli VP, Eisman JA, Frampton RJ, Moseley JM, MacIntyre I, Whitehead R, Martin TJ. 1980 Calcitonin and 1,25-dihydroxy vitamin D receptors in several human breast cancer cell lines. *Cancer Res* 40:4764-4767
 11. Goltzman D, Mitchell J. 1985 Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. *Science* 227:1343-1345
 12. Nakamura A, Yamatani T, Arima N, Yamashita Y, Fujita T, Chiba T. 1992 Calcitonin inhibits the growth of human gastric carcinoma cell line KATO III. *Regul Peptides* 37:183-194
 13. Chakraborty M, Chatterjee D, Kellokompu S, Rasmussen H, Baron R. 1991 Cell cycle-dependent coupling of the calcitonin receptor to different G proteins. *Science* 251:1078-1082
 14. Barsony J, Marx SJ. 1988 Dual effects of calcitonin and calcitonin gene-related peptide on intracellular cyclic 3',5'-monophosphate in a human breast cancer cell line. *Endocrinology* 122:1218-1223
 15. Findlay DM, deLuise M, Michelangeli VP, Ellison M, Martin TJ. 1980 Properties of calcitonin receptor and adenylate cyclase in BEN cells, a human cancer cell line. *Cancer Res* 40:1311-1317
 16. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. 1983 LnCaP model of human prostatic carcinoma. *Cancer Res* 43:1809-1818
 17. Connolly JM, Rose DP. 1990 Production of epidermal growth factor and transforming growth factor- α by the androgen-responsive LnCaP human prostate cancer cell line. *Prostate* 16:209-218
 18. Kadar T, Redding TW, Ben-David M, Schally AV. 1988 Receptors for prolactin, somatostatin, and luteinizing hormone-releasing hormone in experimental prostate cancer after treatment with analogs of luteinizing hormone-releasing hormone and somatostatin. *Proc Natl Acad Sci USA* 85:890-894
 19. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provezano MD, Fujimoto EK, Goede NM, Olson BJ, Klenk DC. 1985 Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76-85
 20. Munson PJ, Rodbard D. 1980 Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 107:220-239
 21. Wiegmann TB, Welling LW, Beatty DM, Howard DE, Vamos S, Morris SJ. 1993 Simultaneous imaging of intracellular [Ca²⁺] and pH in single MDCK and glomerular epithelial cells. *Am J Physiol* 265:C1184-1190
 22. Chabre O, Conklin BR, Lin HY, Lodish HF, Wilson E, Ives HE, Catanzariti L, Hemmings BA, Bourne HR. 1992 A recombinant calcitonin receptor stimulates 3',5'-cyclic adenosine monophosphate and Ca²⁺/inositol phosphate signaling pathways. *Mol Endocrinol* 6:551-556
 23. Shulkes A, Fletcher DR, Rubinstein C, Ebeling PR, Martin TJ. 1991 Production of calcitonin gene-related peptide, calcitonin and PTH-related protein by prostatic adenocarcinoma. *Clin Endocrinol (Oxf)* 94:387-393
 24. Nicholson GC, Moseley GM, Sexton PM, Mendelsohn FAO, Martin TJ. 1986 Abundant calcitonin receptors in rat osteoclasts. *J Clin Invest* 78:355-360
 25. Marx SJ, Aurbach GD, Gavin JR, Buell DW. 1974 Calcitonin receptors on cultured human lymphocytes. *J Biol Chem* 249:6812-6816
 26. Marx SJ, Woodward C, Aurbach GD, Grossman H, Keutmann HT. 1973 Calcitonin receptors on cultured human lymphocytes. *J Biol Chem* 248:4797-4802
 27. Rizzo AJ, Goltzman D. 1981 Calcitonin receptors in the central nervous system of the rat. *Endocrinology* 108:1672-1677
 28. Inaba M, Morii H, Nishizawa Y, Miki T, Yukioka M, Morisawa S, Inoue A. 1986 Calcitonin-induced phosphorylation of rat liver cytosolic proteins. *J Biochem* 100:591-595
 29. Findlay DM, Martin TJ. 1986 Kinetics of calcitonin receptor internalization in lung cancer (BEN) and osteogenic sarcoma (UMR 106-06) cells. *J Bone Mineral Res* 1:277-283
 30. Henke H, Tobler PH, Fischer JA. 1983 Localization of salmon calcitonin binding sites in rat brain by autoradiography. *Brain Res* 272:373-378
 31. Albrant K, Mull E, Brady E, Herich J, Beaumont K. Molecular cloning of two receptors with high affinity for salmon calcitonin from rat brain. 75th Annual Meeting of The Endocrine Society, Las Vegas NV, 1993 (Abstract 1403)
 32. Murphy E, Chamberlin ME, Mandel LJ. 1986 Effects of calcitonin on cytosolic Ca²⁺ in a suspension of rabbit medullary thick ascending limb tubules. *Am J Physiol* 251:C491-C495
 33. Zaidi M, Datta HK, Moonga BS, MacIntyre I. 1990 Evidence that actions of calcitonin on rat osteoclasts is mediated by two G proteins acting via separate post-receptor pathways. *J Endocrinol* 26:473-481
 34. Barsony J, Marx SJ. 1988 Dual effects of calcitonin and calcitonin gene-related peptide on intracellular cyclic 3',5'-monophosphate in a human breast cancer cell line. *Endocrinology* 122:1218-1223
 35. Yanjun S, Chakravarty M, Nathanson MH, Baron R. 1992 Differential effects of the 3',5'-cyclic adenosine monophosphate and protein kinase C pathways on the response of isolated rat osteoclasts to calcitonin. *Endocrinology* 131:1497-1502
 36. Miller JBA, Rozengurt E. 1990. Arachidonin acid release by bombesin: a novel post-receptor target for heterologous mitogenic desensitization. *J Biol Chem* 265:19973-19979
 37. Rozengurt E. 1986 Early signals in the mitogenic response. *Science* 234:161-166

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

BLACK BORDERS

IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

FADED TEXT OR DRAWING

BLURRED OR ILLEGIBLE TEXT OR DRAWING

SKEWED/SLANTED IMAGES

COLOR OR BLACK AND WHITE PHOTOGRAPHS

GRAY SCALE DOCUMENTS

LINES OR MARKS ON ORIGINAL DOCUMENT

REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.